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EXPERIMENTAL STUDY

Effect of Artemisia species on cellular proliferation and apoptosis in human breast cancer cells *via* estrogen receptor-related pathway

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Abstract

OBJECTIVE: To investigate the mechanism underlying the anticancer effect of Artemisia species through the inhibition of cell growth and induction of apoptosis in breast carcinoma cells.

METHODS: To evaluate the anticancer activity of methanol extracts of eight Artemisia species (Artemisia stolonifera, Artemisia selengensis, Artemisia japonica, Artemisia Montana, Artemisia capillaris, Artemisia sylvatica, Artemisia keiskeana, and Artemisia scoparia), we first investigated the proliferation of estrogen receptor (ER)-positive MCF-7 breast carcinoma cells exposed to 5 or 200 g/mL for 72 h. Apoptosis induction was assessed by an Annexin V binding assay in cells exposed to extracts at a high concentration (200 g/mL). To verify the mechanism of apoptosis, ER expression and its related signaling was investigated using an immunoblot assay under the same conditions.

RESULTS: MCF-7 cells showed the strongest antiproliferative response to the tested extracts. However, a biphasic effect was observed: the extracts inhibited proliferation at high concentrations whereas they stimulated it at low ones. ER expression was similarly modulated by the extracts. However, all of the extracts induced apoptosis at a high concentration (200 g/mL). Compared to the control level, exposure to the extracts resulted in a remarkable increase in the shift of cell populations.

CONCLUSION: The present study suggests that the tested Artemisia species exerted their anticancer effects through the induction of apoptosis via an ER-related pathway.

Key words: Artemisia; Breast neoplasms; Bcl-2 gene; Cyclin D1; Estrogen receptor

INTRODUCTION

A great deal of research has been conducted on the use of plants for health-promoting purposes because most people have broad experience with the use of traditional plant-derived products (drug and supplements). Among the numerous herbs used in herbal medicine, growing interest has focused on the use of Artemisia spp. for preventing and reducing the risk of various cancers.[1,2]

We previously showed that Artemisia spp. had significant anticancer, anti-inflammatory, and antiobesity effects *in vitro*.[3] The anticancer activity of Artemisia spp., which involves the inhibition of cellular proliferation, suggested the value of using members of this genus in the production of bioactive food supplements.

Moreover, according to published reports, various

Artemisia spp. have been shown to have activity against several cancer cell types, including hepatoma, gastric cancer, leukemia, and skin cancer cells.[4-7] The results of these studies suggest that the anticancer activity of Artemisia spp. depends on the type of cancer.

Breast cancer is one of the most frequently diagnosed cancers in women, and a few studies have reported that Artemisia preinceps,[7] Artemisia asiatica,[8] Artemisia monosperma[9] and Artemisia argyi[10] have anticancer effects on breast cancer. And some studies have shown that Artemisia preinceps,[8] Artemisia asiatica,[9] Artemisia monosperma,[10] and Artemisia argyi[11] have activities against breast cancer.

According to a report based on the WHO database, an increasing trend in breast cancer mortality was observed in Japan and the Republic of Korea, while breast cancer mortality (1990-2006) in the United States and Europe (UK, Spain, and Germany) showed a decreasing trend. However, the incidence of breast cancer in these populations remains high. Released by US National Cancer Institute, a report estimates that one in eight women in the United States (approximately 13.3%) will develop breast cancer during her lifetime.[12,13] Breast cancer represents a group of heterogeneous diseases with several clinical, molecular, and histopathological forms, which makes achieving effective chemotherapy problematic. Estrogen receptor a (ERa), which is expressed in approximately 70% of breast cancers,[14] makes it difficult to obtain a response to cancer drug treatment.

A need exists to study the targeting of ERa to develop breast cancer therapies. The aim of the present study was to evaluate the anticancer activity of eight Artemisia species against human breast cancer MCF-7 cells and to investigate the underlying mechanism. We found that the Artemisia spp. inhibited cellular proliferation and induced apoptosis via ERa-related pathway.

MATERIALS AND METHODS

Cells culture

The MCF-7 cells were used in the present study. These cells were purchased from Korean Cell Line Bank, Seoul, Republic of Korea. Each cells was routinely maintained in either RPMI 1640 [Roswell Park Memorial Institute medium, Invitrogen (Molecular Probes), Gibco, Carlsbad, CA, USA] or DMEM [Dulbeco's Modified Eagle's Medium, Invitrogen (Molecular Probes), Gibco, Carlsbad, CA, USA], supplemented with 10% Fetal bovine serum and antibiotics (50 U/mL of penicillin and 50 μg/mL streptomycin, Sigma-Aldrich Co., LLC., CA, USA) at 37℃ in a humidified atmosphere containing 5% CO_2. Each cell lines were plated at density of 5-1.0´10^4 cells/well and 3-5´10^5 cells/well on 96-well and 6-well plates for MTT assay and immunoblotting assay, respectively.

Preparation of eight kinds of extract

Abbreviation and voucher specimen number of Artemisia species, which used in present study, was as in the following; Artemisia stolonifera (AST, PB4916.1), Artemisia selengensis (ASE, PB4913.1), Artemisia japonica (AJA, PB4895.1), Artemisia Montana (AMO, PB4920.1), Artemisia capillaris (ACA, PB4894.2), Artemisia sylvatica (ASY, PB4919.1), Artemisia keiskeana (AKE, PB4901.1), and Artemisia scoparia (ASC, PB4893.5). Methanol extracts of Artemisia species were supplied by Herbarium of the KRIBB (The Korea Research Institute of Bioscience & Biotechnology, Daejeon, Korea). Taxonomical identification was confirmed by a botanist and voucher specimens stored at the KRIBB Herbarium. These extracts were diluted in DMSO (dimethyl sulfoxide) to 25 mg/mL just before use.

MTT assay

Cell proliferation was determined using the MTT assay. Each cell type was exposed to each extracts at a concentration of 5-200 μg/mL for 24, 48, and 72 h. After incubations, plated cells were incubated with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma Chemical Company, Perth, Australia, 0.5 mg/mL final concentration) for 4 h at 37℃. After discarding all medium from the plates, 100 μL of DMSO was added to the each well. The plates were placed for 5 min at room temperature with shaking, so that complete dissolution of formazan was achieved. The absorbance of the MTT formazan was determined at 540 nm by a UV spectrophotometric plate reader (Molecular Devices, LLC., CA, USA). From the plot estimated was the value of IC_{50} (i.e., the concentration of the extract required to inhibit cancer cell growth by 50% of the control level, that is, each cell was treated with only compound solvent).

Apoptosis detection assay

Annexin V-FITC apoptosis kit (BD ApoAlert ™, BD Biosciences Clontech, CA, USA) was used for apoptosis detection. Cells were trypsinized, washed twice in ice-cold PBS, and resuspended in 500 μL binding buffer (Sigma-Aldrich Co., LLC., CA., USA). Annexin V and propidium iodide solution were added to the cell preparations and incubated for 25 min in the dark. Binding buffer (400 μL) was then added to each tube and the samples were analyzed by a FACS Calibur instrument (BD Biosciences Clontech, CA, USA) equipped with CellQuest 3.3 software (BD Biosciences, CA, USA).

Immunoblotting assay

Each cell type was exposed to each extract for 72 h at two concentrations (10 or 200 mg/mL). Then after, cells were lysed in radioimmunoprecipitation assay buffer (RIPA buffer; 1% nonidet P-40, 150 mM NaCl, 0.05% deoxycholic acid, 1% sodium dodecyl sulfate, 50 mM Tris, pH 7.5) containing protease

inhibitor for 1 h at 4℃. The supernatant was separated by centrifugation, and protein concentration was determined by Bradford protein assay kit II (Bio-rad Laboratories, CA, USA). Proteins (25 μg/well) denatured with sample buffer were separated by 10%-12% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes (0.45 μm). The membranes were blocked with a 1% BSA (bovine serum albumin) solution for 3 h and washed twice with PBS containing 0.2% Tween-20, and incubated with the primary antibody overnight at 4℃.

Antibodies against estrogen receptor-a (ERa), cyclin D1, Bcl-2, Bax and b-actin were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc., CA., USA) and used to probe the separate membranes. The next day, the immunoreaction was continued with the secondary goat anti-rabbit horseradish-peroxidase-conjugated antibody after washing for 2 h at room temperature. The specific protein bands were detected by Opti-4CN Substrate kit (Bio-rad Laboratories, CA, USA).

Statistical analyses

All data were expressed as percent compared with vehicle-treated control cells, which were arbitrarily assigned 100%. Data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test (SigmaStat 3.1 statistical program, Systat Software Inc., CA, USA). For all comparisons, differences were considered statistically significant at $P<0.05$.

RESULTS

Antiproliferative effects of Artemisia extracts

We first examined the possible antiproliferatative effects of different Artemisia extracts (AST, ASE, AJA, AMO, ACA, ASY, AKE, and ASC) on ERa-positive MCF-7 cell lines. When cell lines were exposed to various concentrations of extracts (5-200 mg/mL) for 72 h (Figure 1), the antiproliferative effects of the extracts were stronger in MCF-7 cells. However, exposure to all extracts apart from the AST and ASE extracts at concentrations less than 10 mg/mL stimulated the proliferation of MCF-7 cells. Cell growth was increased by 12% and 17% in MCF-7 cells treated with ASE and AJA, respectively, compared to controls, although the differences were not statistically significant. Increases in the others were 5%-10% . When applied at concentrations above 10 mg/mL, all extracts significantly inhibited proliferation in a dose-dependent manner ($P<0.05$). The response was not greatly affected by exposure time (within a 12%-25% decrease in cells exposed to longer treatment, compared to those exposed to the extracts for 24 h). Notably, treatment with the ASE, AKE, and ASC extracts at a concentration of 200 mg/mL for 72 h reduced cell proliferation by 59.1%, 57.3%, and 64.7%, respectively, compared to controls. The AST and ASE extracts, when applied at higher concentrations

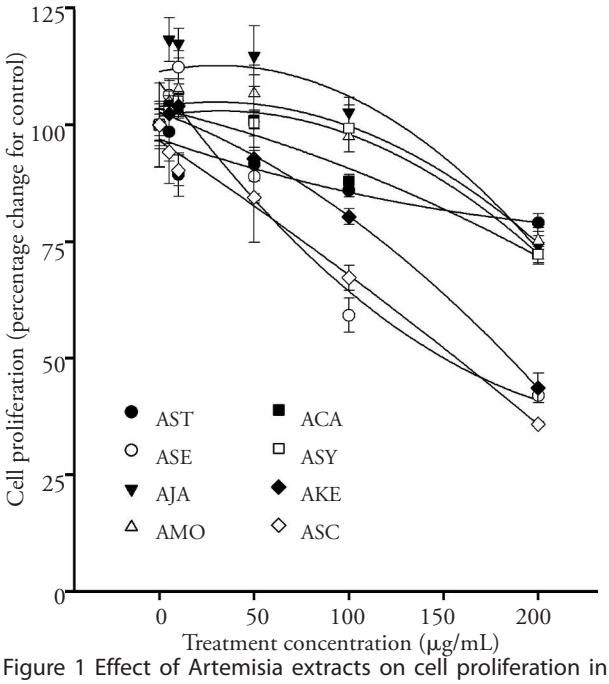

Figure 1 Effect of Artemisia extracts on cell proliferation in estrogen receptor-a positive human breast MCF-7 cells

AST: Artemisia stolonifera; ASE: Artemisia selengensis; AJA: Artemisia japonica; AMO: Artemisia montana; ACA: Artemisia capillaris; ASY: Artemisia sylvatica; AKE: Artemisia keiskeana; ASC: Artemisia scoparia. Cells were exposed to each extracts at a concentration of 5-200 μg/mL for 72 h. All data are reported as the percentage change in comparison with the vehicle-only group.

(200 mg/mL for 72 h), inhibited proliferation by 27.5% and 36.7%, respectively, compared to controls. The other extracts showed approximately 15%-20% inhibition.

Apoptosis induction by Artemisia extracts

To investigate Artemisia-induced apoptosis in MCF-7 cells, cells were treated with 200 mg/mL Artemisia extract for 72 h (Figure 2). Compared with the control, the number of apoptotic cells was increased significantly by all of the tested extracts. As a similar pattern of antiproliferative activity, treatment with ASE, AKE, and ASC extracts increased the number of apoptotic cells by 53.9%, 61.3%, and 55.4%, respectively, compared to the controls.

Effects of Artemisia extracts on ERa, cyclin D1 and Bcl-2 expression and apoptosis induction

ERa expression was observed in MCF-7 cells exposed to extracts (10 or 200 mg/mL) for 72 h (Figure 3). ERa expression by MCF-7 cells exposed to extracts showed a pattern similar to their antiproliferative effects; that is, stimulation by lower some extracts (ASE, AJA, AMO and ACA) concentrations was detected. However, all extracts at higher concentrations (200 mg/mL) reduced ERa expression. Moreover, all extracts induced apoptosis in MCF-7 cells exposed to 200 mg/mL concentration through downregulation of cyclin D1 and Bcl-2 expression.

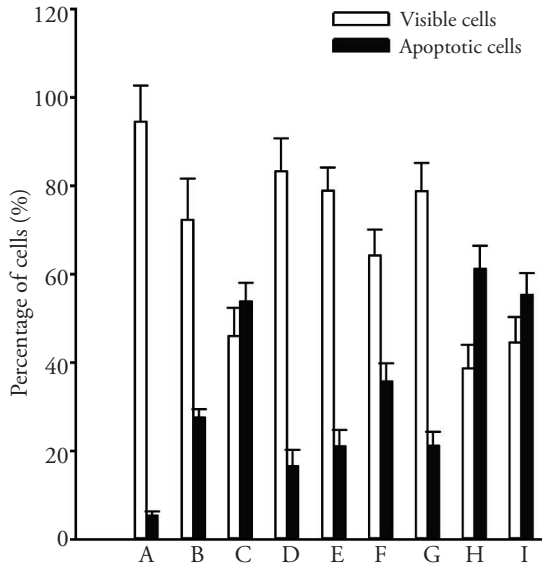

Figure 2 Effect of Artemisia extracts on apoptosis induction in estrogen receptor-a positive human breast MCF-7 cells
A: control; B: AST (Artemisia stolonifera); C: ASE (Artemisia selengensis); D: AJA (Artemisia japonica); E: AMO (Artemisia Montana); F: ACA (Artemisia capillaris); G: ASY (Artemisia sylvatica); H: AKE (Artemisia keiskeana); I: ASC (Artemisia scoparia). Cells were exposed to each extracts at a concentration of 200 μg/mL for 72 h.

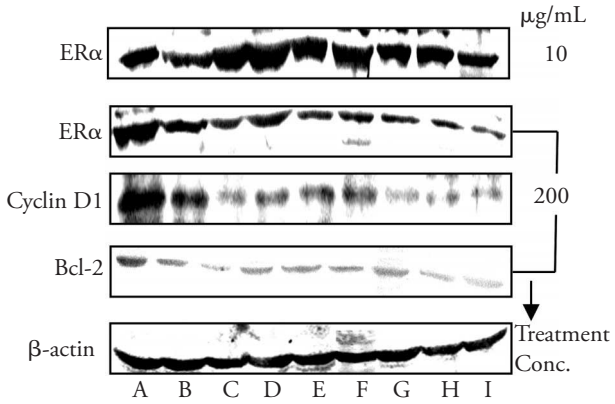

Figure 3 Effect of Artemisia extracts on estrogen receptor-a (ERa, 66kDa), cyclin D1 (38 kDa), and Bcl-2 (26 kDa) expression in ER-a positive human breast MCF-7 cells
A: control; B: AST (Artemisia stolonifera); C: ASE (Artemisia selengensis); D: AJA (Artemisia japonica); E: AMO (Artemisia Montana); F: ACA (Artemisia capillaris); G: ASY (Artemisia sylvatica); H: AKE (Artemisia keiskeana); I: ASC (Artemisia scoparia). Conc.: concentration. To investigate ERa expression, cells were exposed to each extract for 72 h at two concentrations (10 or 200 mg/mL) to stimulate or inhibit proliferation. In addition, cyclin D1 and Bcl-2 expression levels were determined in cells exposed to each extract at a concentration of 200 mg/mL for 72 h.

DISCUSSION

Natural phytochemicals have been accepted as a type of medicine, and epidemiological and experimental studies have demonstrated that traditional herbs can reduce the incidence of certain forms of cancer.[15-18] Based on a study showing that Artemisia spp. have beneficial biological activities, including anticancer, antiobesity, and anti-inflammatory activities, we investigated the anticancer mechanism of Artemisia spp. in the present study.

The antiproliferative effects of Artemisia extracts on ERa-positive T47D cells at high concentrations (> 100 mg/mL) for 72 h were significantly greater than those of the extracts on ERa-negative HS578T cells, although the extracts stimulated T47D cell growth at lower concentrations and shorter times.[3] This result is consistent with those of the present study in that a biphasic effect was observed, with the extracts inhibiting proliferation at high concentrations and stimulating it at low ones. When we investigated the anticancer activity of methanol extracts of eight Artemisia spp. (AST, ASE, AJA, AMO, ACA, ASY, AKE and ASC) against ERa-positive MCF breast carcinoma cells at a concentration of 10 or 200 mg/mL for 72 h, MCF-7 cells showed the strongest antiproliferative response to the tested extracts at high concentrations but also exhibited growth at low concentrations.

These data suggest that the antiproliferative activity of Artemisia extracts involves an ERa-related pathway. ERa is a member of the steroid receptor superfamily that regulates processes such as growth and differentiation in various target cells by regulating transcription.[19] ERa also plays an important role in the development and progression of breast cancer.[20] The results of several recent epidemiological studies suggest that phytoestrogens, plant-derived phenolic compounds that structurally mimic the hormone 17β-estradiol,[21] may reduce the risk of cancer. A new strategy for breast cancer chemotherapy has been targeted on ERa regulators among phytoestrogens. On the other hand, previous studies suggest that estrogen-like bioactive molecules may stimulate breast cancer cell growth.[22-24] Also, some drugs (e.g., tamoxifen, a representative selective estrogen receptor modulator) act as ER agonists, and this ER-agonistic activity may be associated with an increased risk of endometrial cancer.[25]

Although it was not a significant effect, the Artemisia extracts induced MCF-7 cell growth at low concentrations (<10 mg/mL). This may be explained by the fact that the genus Artemisia is comprised of polyphenol-rich plants.[26,27] Depending on their polyphenol bioactive molecule content, Artemisia extracts seem to have weak ER-agonist activity. This is consistent with our data showing that the extracts significantly reduced ERa expression at higher concentration (200 mg/mL), but that some (ASE, AJA, AMO, and ACA) induced it at a lower concentration (10 mg/mL). The observed antiproliferative activities of Artemisia are encouraging, but its use should be carefully monitored. The Artemisia extracts reduced cyclin D1 expression when applied to cells at a high concentration. Cyclin D1 is a well-known target of estrogens in breast cancer cells, and its induction is important for the progression of cells through the G1 phase of the cell cycle.[28] The results of several studies

suggest that cyclin D1 is overexpressed in breast cancer, [29,30] and that it is associated with ER positivity in breast cancer.[31-33] These results suggest that the regulation of cellular proliferation in MCF-7 cells by Artemisia extracts was related to ERa signaling.

Moreover, the induction of apoptosis by the Artemisia extracts is supported by evidence that the rate of apoptotic cells was increased in MCF-7 cells exposed to high concentrations of extract. Apoptosis (i.e., programmed cell death) is essential for tissue development and homeostasis. The mechanism of apoptosis involves a balance between factors that induce and those that inhibit the process. Recently, pro-apoptotic agents have been proposed for cancer chemotherapy.

Much effort has been devoted to the search for new agents for the therapy, and several traditional plant-derived products have been found to be potentially useful. The anticancer activities of Artemisia spp. (e.g., AST, AKE, and ASC), demonstrated in the present study, make them hypothetically useful for further development as a chemotherapeutical agent targeted at ERa-related signaling pathway in human breast cancer.

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